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RESEARCH REPORT

Heat shock transcriptional factors (HSFs) are expressed in response to hydrogen peroxide production in grapevines inoculated with *Colletotrichum* Species

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Abstract
Heat shock transcriptional factors (HSFs) are expressed in plants due to external stress, high temperatures, or pathogenic infections. This study was undertaken to analyze the expression of HSF genes in grapevines inoculated with pathogens. Spores of *Colletotrichum acutatum* and *C. gloeosporioides* were inoculated on the leaves, and on immature and mature fruits of 'Campbell Early' and creeping grapevine (*Vitis flexuosa*). The real-time PCR of RNA isolated from infected leaves and fruits showed specific upregulation of HSF11, HSF12, HSF14, and HSF15 subsequent to both high temperature and pathogen infections. However, HSF16 and HSF17 were up-regulated specifically by pathogenic infections only and showed different expression patterns in response to wounding. Among the hydrogen peroxide-related genes, the expression of catalase (CAT) and superoxide dismutase (SOD) was up-regulated by pathogen infections. Concurrently, expression of six HSFs (HSF11, HSF12, HSF14, HSF15, HSF16, and HSF17) dose-dependently increased with accumulation of hydrogen peroxide in the leaves and fruits of grapevines. The expression of selected HSF genes was up-regulated differentially as a defense reaction in 'Campbell Early' and *V. flexuosa* grapevine in response to external stress (such as wounding) and pathogen inoculation. This indicates that expression of some HSFs is regulated through the hydrogen peroxide-mediated pathways in response to pathogens. Further studies determining the mechanism of HSF gene expression induced by external stress are required.

Keywords Heat shock protein · Hydrogen peroxide · Temperature changes · *Vitis flexuosa*

1 Introduction

Due to the recent climatic changes, the 30 years' average annual temperature and precipitation in Korea have increased by 0.4℃ and 1384.33 mm, respectively (Lee et al. 2007). Changes in weather negatively affect the quality (skin color, total soluble solids, and aroma) and harvest time of grapes as well as wine production (Orduña et al., 2010). Close to 142.4 thousand tons grapes are produced in 12,676 cultivated areas in Korea (KOSIS, 2019). The main grape cultivars are 'Campbell Early', 'Kyoho', 'Shine Muscat', Muscat Bailey A', and 'Delaware', which are consumed mainly as table grapes and processed foods, including wine and jam (Park et al. 2010).

It is well documented that the coloring of grape skins is poor at high temperatures, which causes abnormal production of anthocyanin pigments and subsequently results in low-quality fruits (Shinomiya et al. 2015). Extremely high temperatures are one of the stresses in plant growth and development, causing the plant to activate numerous transcriptional factors to protect themselves from this stress factor (Akhtar et al. 2012; Li et al. 2011; Scharf et al. 2012). Of these, the heat shock transcriptional factors (HSFs) are known to play important roles in plants, including Arabidopsis, tomato, castor, grape, soybean, poplar, and slender false brome (Liu et al. 2018; Scharf et al. 2012). HSFs reportedly regulate the expression of genes such as HSP and APX2 (von Koskull-Doringet al. 2007).

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Table 1 Primer list of VHSF (grapevine HSF) and nucleotide sequences used for real-time PCR analysis (Lee et al. 2021)

Name	Primer sequences (5'-3')	
	Forward	Reverse
VHSF1	CCTGCTCCTTTCCTGTTGAAGA	ACAAACCCTGTTCCATCTGCAT
VHSF2	CTCTTGGAGCAGAGCAGGTAGC	AAGTTTTTGGGGAGGAGATTGG
VHSF3	ATAGATGGGAGTTTGCCAACGA	AGTGGAGAATGGTGATGGTGGT
VHSF4	GATGGGAATTCTCCAACGACTG	ATCGTTCGTCAGCACAATTTGA
VHSF5	AGGCAAAAATATGCTGGAACGA	TGGAATTGGTAGAGGCTTTGGA
VHSF6	AATTCGATCGTGTCCTGGAGTC	CGGTAAAAGGTCTCTTGCGAAC
VHSF7	CCAAAGAGGAGAAGATGCTGGA	ACAACACCATCCACTCTCACCA
VHSF8	GATCCAATCGACATCGTCTTCC	ACCTACAGTGCTGCCGTCTTCT
VHSF9	GATCTTTGCCGGGAATCTTCTT	GCAAACTCCCATCTGTCTGGAT
VHSF10	AGAGGTGAAATCAGAGCCGTTG	AGTTCTAGCCACGGAGGGTCTT
VHSF11	CTGAGTTCGCCAAAGATTTGCT	CTCAGGATCAGCCTTCCTGAAA
VHSF12	CAAGGTCAAAGAGCAGCAGTCA	AGGGTTCTGAACTGCCTTTTCC
VHSF13	TGCAAATGCAGCATCTAAAGGA	ACAGTTTGCTGACGTTGTTCCA
VHSF14	GAGGACCCCAATGAGGAGAGTT	CCCCTTTTCTTCTTTCCCTTCA
VHSF15	TGCCCAAGTACTTCAAGCACAA	TGGATCCACCTTCCTAAATCCA
VHSF16	CACAGCAACTTCTCCAGCTTTG	CTCTTTCCCCTCTTCGGAACAT
VHSF17	GTTCACAACCATTCCCTTCTGG	CAATGGGTTCACCTGAGAAAGG
VHSF18	ATTGCTGGCAATGAGGAAGAAG	ATGATGATCACCCAGTCGGAGT
VHSF19	CTGGATTGATTCCCCATTACCA	CGGTGGTTATCTTGGGTGAGAG

Although HSFs are expressed by abiotic stresses including high temperatures, their expression in response to biotic stress (including infection of pathogens) remains unknown. Among the various plant diseases, the occurrence of grey mold caused by *Botrytis cinerea*, peduncle rot by *Botryosphaeria dothidea*, ripe rot by *Colletotrichum gloeosporioides* and *C. acutatum*, bird's-eye rot (anthracnose) by *Elsinoe ampelina*, downy mildew by *Plasmopara viticola*, leaf spot by *Pseudocercospora vitis*, and powdery mildew by *Uncinular necator* has been reported in Korean vineyards (Hong et al., 2008; Lim et al. 2017). Ripe rot by *C. acutatum* and *C. gloeosporioides* causes symptoms such as ring spot or fruit cracking, and it is known that European grapes with thin skins are more susceptible to ripe rot compared to American grapes. When plants are exposed to biotic stresses (such as pathogens like the *Colletotrichum* species) and abiotic stresses (such as high temperature and drought), reactive oxygen species (ROS) are generated inside the plant to exert a defense reaction in response to the stresses (Das and Roychoudhury 2014; Mehdy 1994).

The expression of HSF genes in grapevines has been reported to be induced through the accumulation of ROS in plants exposed to abiotic stress (Miller and Mittler 2006), but there is little information on how expression is affected by pathogenic infections. Therefore, this study was undertaken to investigate the expression of HSF genes and to elucidate the mechanism of this expression through accumulation of ROS in grapevines after pathogen infections.

Table 2 Primer list of hydrogen peroxide–related genes and nucleotide sequences used for real-time PCR analysis

Name	Primer sequences (5'-3')	
	Forward	Reverse
VvMnSOD	ATAACCAACTACAA-CAAAGCCCTA	CTTTCCATAC-GTTCTTCAG-GTAAT
VvAPX	GGTCCGTTTGGGACAATGAA	CGGAAATT-GCTCCTT-GATCG
VvCAT	TCATGCTACTCAGGATCTC-TATGA	CTTGAAATT-GTTCTCCTTCT-CAAT

Vv: *Vitis vinifera*, MnSOD: manganese superoxide dismutase, APX: ascorbate peroxidase, CAT: catalase

2 Materials and methods

2.1 Plant materials and pathogens

'Campbell Early' (*Vitis labruscana*) and the creeping grapevine native to Korea (*V. flexuosa*), which were kept in greenhouses and *Vitis* germplasm vineyards at Yeungnam University, were used as the plant materials in this study. Two pathogens, *C. acutatum* and *C. gloeosporioides*, were used for inoculating the grapevine leaves and grape berries.

2.2 Pathogen culture and inoculation

Pathogens were cultured in potato dextrose agar (PDA) medium under a wavelength of 380 nm (Anderson et al. 2013), in a 12-h-light/12-h-dark cycle to induce sporulation. Spore suspension (1×1^6 spores/mL) was prepared

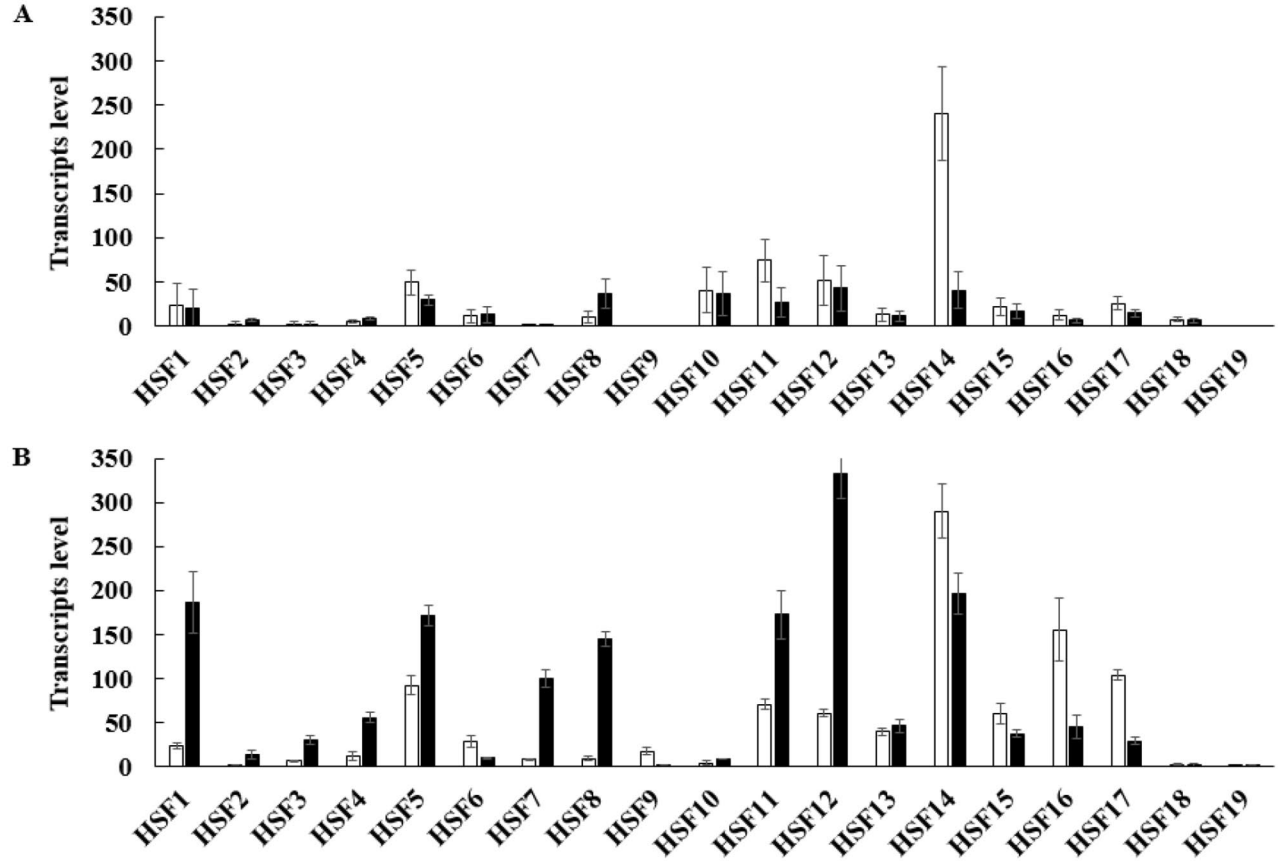

Fig. 1 Expression levels of VHSFs (grapevine heat shock transcriptional factors) at 6 h after treatment in leaves of 'Campbell Early' (□) and creeping grapevine (*V. flexuosa*, ■) in response to pathogen and high temperature. 'Campbell Early' grapevines and creeping grapevines were exposed to 35℃ (A) and inoculated with *Colletotrichum acutatum* (B). Vertical bars represent standard error of means ($n = 3$)

from the cultured pathogens using sterile water. Inoculation was achieved using a nebulizer, spraying onto the leaves, immature fruit, and mature fruit of 'Campbell Early' and creeping grapevine. Samples inoculated with pathogens were collected at varying time points, rapidly frozen with liquid nitrogen, and stored in a deep freezer at -80℃.

2.3 RNA isolation and real-time PCR

RNA was isolated from the leaves, immature, and mature fruits of 'Campbell Early' and creeping grapevine inoculated with pathogens, applying the method described by Chang et al. (1993). A Nano Drop spectrophotometer (NABI, UV spectrophotometer, Korea) was used to measure the RNA yield and quality. cDNA was synthesized from 500 ng RNA using the GoScript™ Reverse Transcription System (Promega, Madison, USA), and real-time PCR was performed using this cDNA as the template. SYBR Premix Ex (SYBR Premix Ex Taq, TaKaRa Bio Inc., Osaka, Japan) was used for real-time PCR. The reactions were subjected to one cycle at 95℃ for 30 s, followed by 40 cycles at 95℃ for 5 s and at 60℃ for 30 s. The nucleotide sequences of primers used from 19 grape HSF genes and ROS-related genes (including catalase (CAT), superoxide dismutase (SOD), and ascorbate peroxidase (APX)) are listed in Tables 1 and 2. All qPCR experiments were performed in triplicate to ensure consistency of the results, using gene-specific primers designed to target each gene of interest.

2.4 Measurement of hydrogen peroxide (H_2O_2)

The concentration of hydrogen peroxide (H_2O_2) was determined following the method of Loreto and Velikova (2001). Briefly, leaf samples (0.3 g) were homogenized in 3 mL 1% (w/v) trichloroacetic acid (TCA), followed by centrifugation of the homogenate at 10,000 *g* and 4℃ for 10 min. Subsequently, 0.75 mL of the supernatant was added to 0.75 mL 10 mM K phosphate buffer (pH 7.0) and 1.5 mL 1 M KI, and absorbance was measured at 380 nm. The concentration of H_2O_2 was calculated from a standard curve plotted in the range 100 to 1000 mmol/mL and is expressed as µmol/g FW.

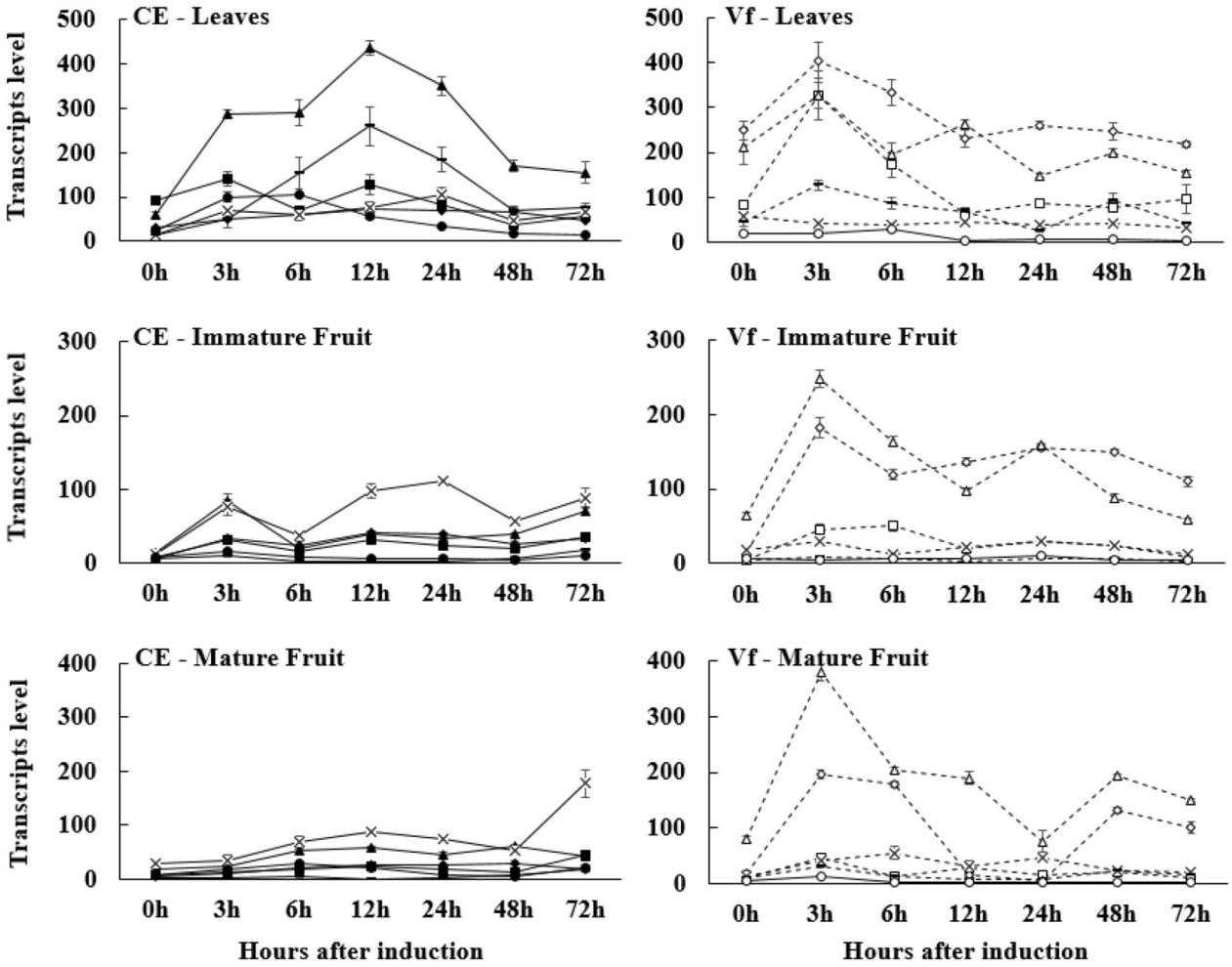

Fig. 2 Expression levels of HSF11 (heat shock transcriptional factor) (□), HSF12 (◇), HSF14 (△), HSF15 (x), HSF16 (-), and HSF17 (○) genes in leaves and immature and mature fruits of 'Campbell Early' (CE, —) and creeping grapevine (*V. flexuosa*, Vf, ---) in response to *Colletotrichum acutatum*. Vertical bars represent standard error of means ($n = 3$)

3 Results and discussion

3.1 Selection of HSFs differentially expressed by pathogens

Real-time PCR was performed using primers designed from HSF1 ~ 19 nucleotide sequences, made using cDNA designed from the RNA isolated from leaves of 'Campbell Early' and creeping grapevine inoculated with pathogens and exposed to high temperature (30–35°C).

Incubation in a 30°C moist chamber for 3 days after inoculation with *Colletotrichum* pathogens resulted in the typical symptoms in leaves and fruits of both 'Campbell Early' and creeping grapevines.

Evaluating the expression of HSFs in leaves inoculated with the pathogen and exposed to high temperature revealed the upregulation of four HSFs (HSF11, HSF12, HSF14, and HSF15) in both the pathogen-infected and high temperature–exposed leaves, whereas two HSFs (HSF16 and HSF17) showed differential expression patterns in grapevine leaves under the same conditions (Fig. 1).

Real-time PCR with selected HSFs in grapevines showed that the gene expression levels were higher in most leaves compared to immature or mature fruits of 'Campbell Early' and creeping grapevine inoculated with *C. acutatum*. The expression of HSF11, HSF12, and HSF14 in creeping grapevine was observed to be higher than in 'Campbell Early', while the expression of HSF15, HSF16, and HSF17 in 'Campbell Early' was greater than in creeping grapevine. Except HSF15, the expression levels of all HSFs were the highest at 12 h after inoculation of the pathogen *C. acutatum* (Fig. 2).

The expression of HSF11, HSF12, HSF14, HSF15, HSF16, and HSF17 in the leaves, immature fruit, and mature fruit of creeping grapevine inoculated with *C.*

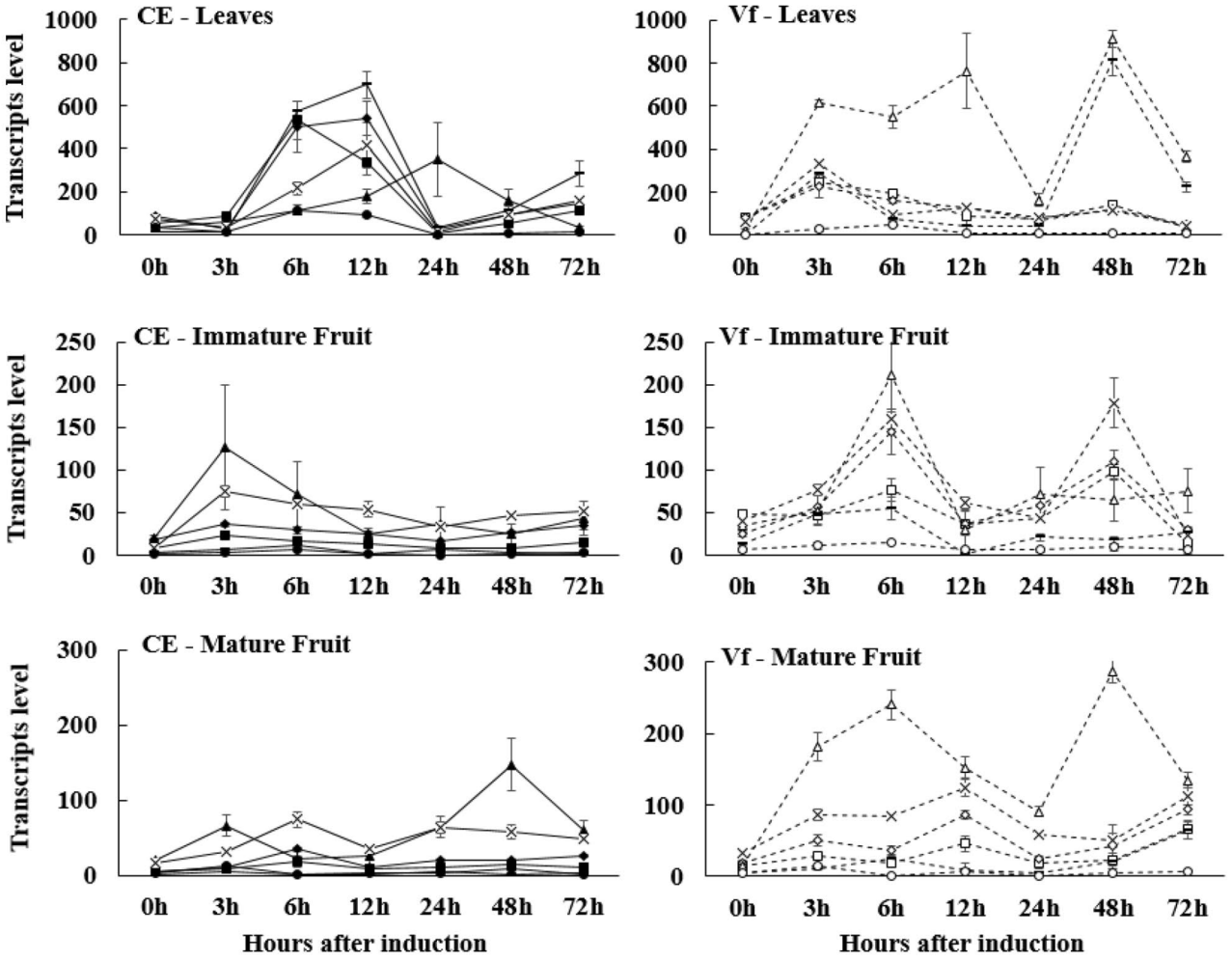

Fig. 3 Expression levels of HSF11 (heat shock transcriptional factor) (□), HSF12 (◇), HSF14 (△), HSF15 (x), HSF16 (-), and HSF17 (○) genes in leaves and immature and mature fruits of 'Campbell Early' (CE, —) and creeping grapevine (*V. flexuosa*, Vf, ---) in response to *Colletotrichum gloeosporioides*. Vertical bars represent standard error of means ($n = 3$)

gloeosporioides was higher than values obtained in 'Campbell Early' grapevine. In addition, the expression was higher in leaves than in immature or mature fruits, and expression levels of most HSFs was highest at 12 h after inoculation of the pathogen *C. gloeosporioides* (Fig. 3). Among the six selected HSFs, HSF16 and HSF17 showed the low expression levels in both 'Campbell Early' and creeping grapevine inoculated with pathogens.

In addition, the expression of HSF14, HSF16, and HSF17 was down-regulated by inoculation of the pathogen compared to levels obtained by wounds in grapevines (Fig. 4).

In plants, HSFs are known to be expressed specifically by abiotic stresses (including drought, salt, CO_2, and high temperature) and are also known to be expressed by biotic stresses (such as fungal and bacterial infections). Although various studies have reported that HSFs are expressed by abiotic stress, the expression of HSFs in response to biotic stress, including infection with pathogens, has not been reported in grapevines.

Therefore, this study investigated the expression levels of VfHSF1 ~ 19 genes in the leaves and in immature and mature fruits of 'Campbell Early' (*V. labruscana*) and creeping grapevine (*V. flexuosa*) inoculated with ripe rot pathogens. Among the HSF genes with differential expression patterns following exposure to high temperature and inoculation with pathogens, HSF11, HSF12, HSF14, HSF15, HSF16, and HSF17 were selected for further studies.

Creeping grapevine (*V. flexuosa*) is native to Korea and is known to be less sensitive to stress by high temperature, thus imparting good coloration of fruit skins at high temperatures (Ahn et al. 2019; Lee et al., 2020). The expression of HSF11 in grapevines inoculated with *C. acutatum* showed a similar reaction in the immature fruit of the creeping grapevine and leaves of 'Campbell Early' exposed to 35 °C. The

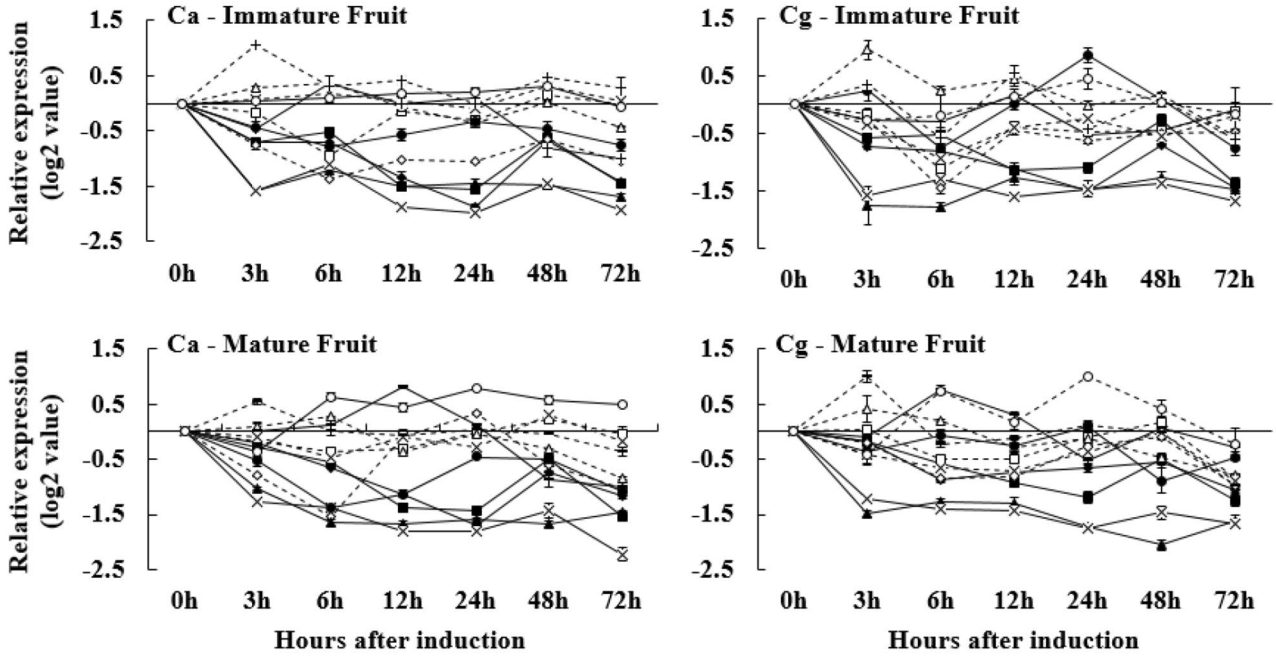

Fig. 4 Relative expression of HSF11 (heat shock transcriptional factor) (■, □), HSF12 (⬥, ◇), HSF14 (▲, △), HSF15 (x, x), HSF16 (+, +), and HSF17 (●, ○) genes in immature and mature fruits of 'Campbell Early' (—) and creeping grapevine (*V. flexuosa*, ---) in response to *Colletotrichum acutatum* (Ca) and *C. gloeosporioides* (Cg). Vertical bars represent standard error of means ($n = 3$)

expression of HSF genes due to pathogen inoculation was higher mostly in the leaves than in fruits and was higher in the creeping grapevine than in the 'Campbell Early' grape. The expression of HSF16 in grapevines inoculated with *C. gloeosporioides* showed a similar reaction with creeping grapevine leaves and 'Campbell Early' leaves exposed to 45 °C, which is similar to a previous report stating that HSF16 shows different reactions in response to high temperature in 'Campbell Early' and creeping grapevine (Lee et al., 2020).

In walnuts, it has been reported that HSF9, HSF10, HSF11, HSF12, HSF13, and HSF17 are expressed differentially in response to abiotic stresses such as high temperature, drought, and salt stress (Liu et al. 2020). HSF5, HSF15, HSF16, HSF17, and HSF18 were also reported to be differentially expressed in canola by abiotic stresses such as drought, high temperature, and high CO_2 (Zhu et al. 2017). HSF5, HSF7, HSF9, HSF12, HSF13, and HSF17 were reported to be expressed by drought stress in sesame seeds (Dossa et al. 2016). In tomatoes, HSF14, belonging to the HSFB group, is known to be expressed in cooperation with the HSFA group (Bharti et al. 2004). Additionally, among the HSF genes, HSF3 showed the highest expression at 6 h after inoculation with *Xanthomonas axonopodis* pv. *manihotis* (Xam) in cassava plants (Wei et al. 2018).

3.2 Measurement of hydrogen peroxide content in grapevines

Hydrogen peroxide is one of the ROSs generated under stress conditions, and the levels were measured in leaves and fruits of 'Campbell Early' and creeping grapevine inoculated with pathogens. The amount of hydrogen peroxide was highest at 3 h post inoculation in leaves and in immature and mature fruits of creeping grapevine inoculated with *C. acutatum.* In 'Campbell Early' inoculated with *C. acutatum*, low levels of hydrogen peroxide were generated in leaves and fruits compared to in creeping grapevine. In the creeping grapevine, the amount of hydrogen peroxide peaked at 3 h in leaves and 6 h in fruits after inoculation with *C. gloeosporioides*. The amount of hydrogen peroxide generated in 'Campbell Early' inoculated with *C. gloeosporioides* was also less than in creeping grapevine inoculated with the same pathogen. The generation of hydrogen peroxide was the highest at 3 and 6 h after inoculation with *C. acutatum* and *C. gloeosporioides*, respectively, in the leaves and immature and mature fruits of 'Campbell Early' and creeping grapevine, and the generation of hydrogen peroxide after pathogen inoculation was determined to be higher in the creeping grapevine than in 'Campbell Early' (Fig. 5).

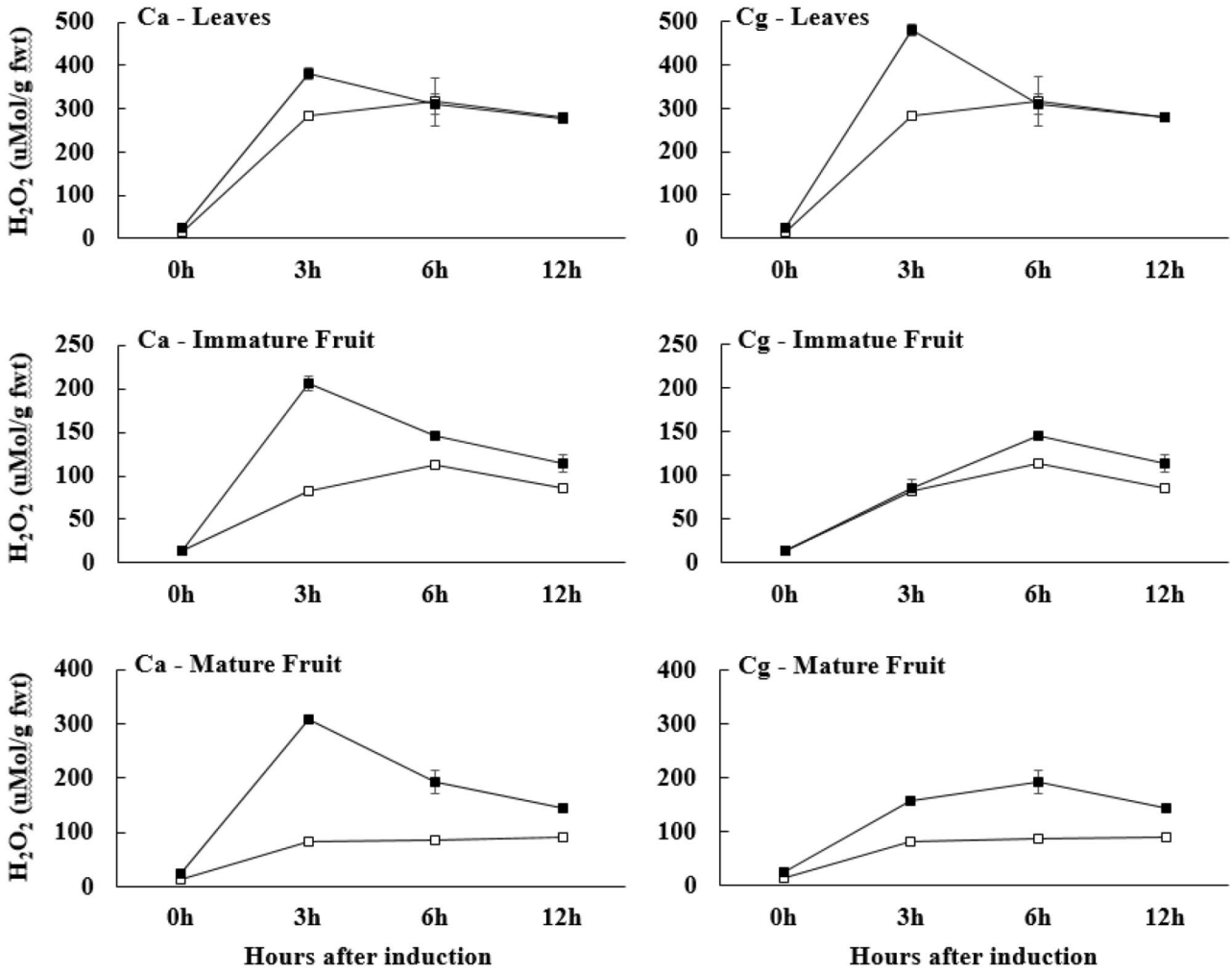

Fig. 5 The amount of hydrogen peroxide in leaves and immature and mature fruit of 'Campbell Early' (□) and creeping grapevine (*V. flexuosa*, ■) inoculated with *Colletotrichum acutatum* (*Ca*) and *C. gloeosporioides* (*Cg*). Vertical bars represent standard error of means ($n = 3$)

3.3 Expression of hydrogen peroxide-related genes

The expression levels of genes related to the generation and elimination of hydrogen peroxide were investigated to predict the role of ROS in response to stresses in grapevines. The ROS-related genes, including superoxide dismutase (SOD) involved in hydrogen peroxide accumulation ($H_2O_2^- + 2\ H^+ \rightarrow H_2O_2 + O_2$), catalase (CAT), and ascorbate peroxidase (APX), were evaluated for their expression.

The expression levels of CAT genes in the leaves and immature and mature fruits of 'Campbell Early' and creeping grapevine inoculated with pathogens were higher than those of SOD and APX. Moreover, the expression levels of ROS-related gene were determined to be higher in creeping grapevine than in the 'Campbell Early' grapevine (Fig. 6). Expression patterns were observed to be dependent on the species of *Colletotrichum*, the ripe rot pathogen in grapevines. The expression of CAT and SOD genes continued to increase in the mature fruit of creeping grapevine, leaves of 'Campbell Early', and creeping grapevine inoculated with *C. acutatum*. However, the increase was not continuous, and levels began to decrease from 3 to 6 h after inoculation in the leaves and fruits of 'Campbell Early' and creeping grapevine inoculated with *C. gloeosporioides*.

HSFs are expressed not only directly in response to foreign biotic and abiotic stresses (Hwang et al. 2014; Li et al. 2014; Liu et al. 2020; Peng et al. 2013; Tanabe et al. 2016; Wei et al. 2018; Zhu et al. 2017), but also through the activation of the ROS accumulation and scavenging pathway (Miller and Mittler 2006; Volkov et al. 2006). ROS increase due to abiotic and biotic stresses up-regulates the gene expression of various enzymes, including SOD, CAT, and APX (Gupta et al. 1993; Luis et al. 2006; Mittler et al., 2004; Moller, 2001). It has been reported that the activity of

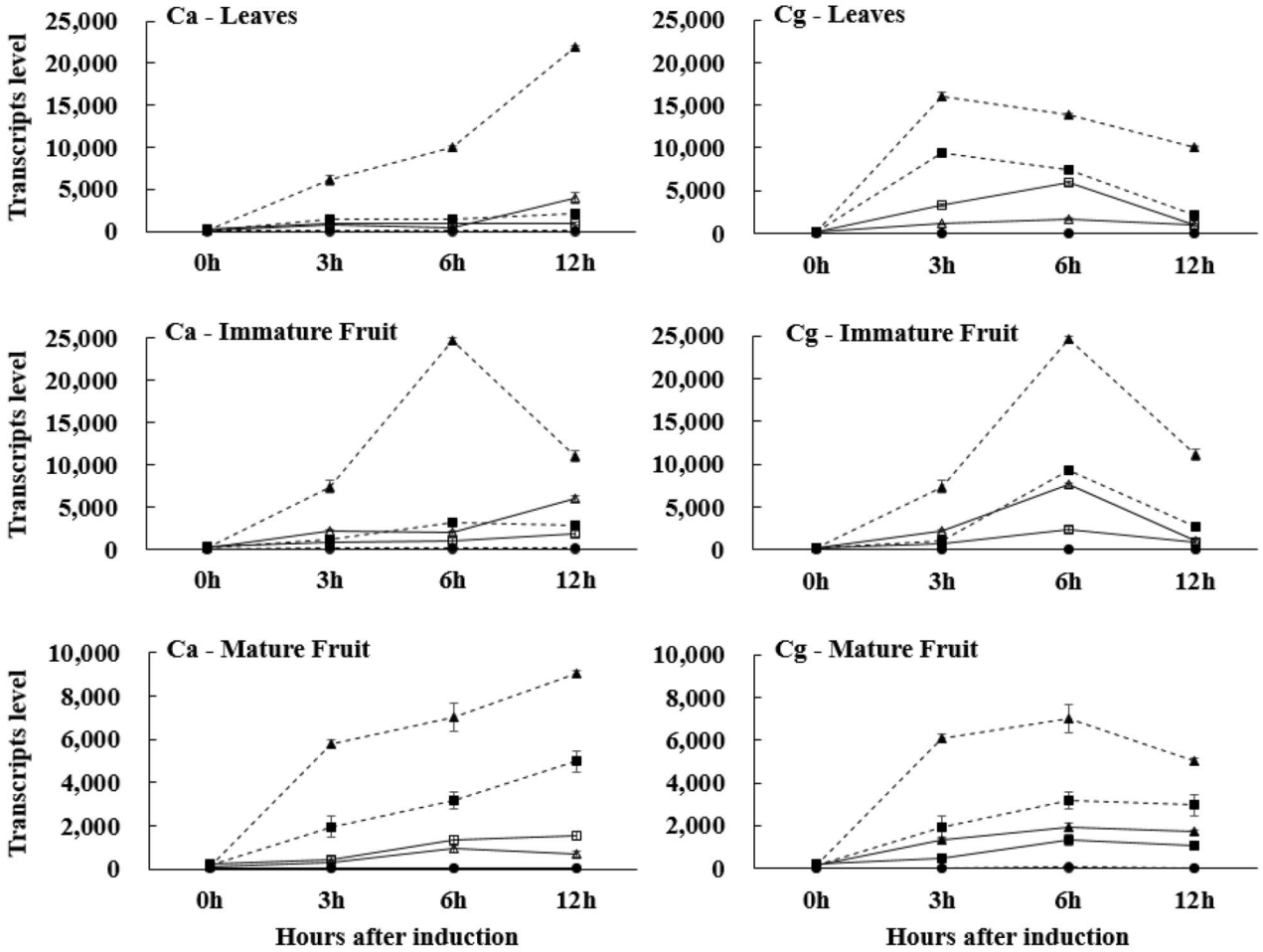

Fig. 6 Expression levels of ascorbate peroxidase (APX: ○, ●), superoxide dismutase (SOD: □, ■), and catalase (CAT: △, ▲) genes in leaves and immature and mature fruit of 'Campbell Early' (—) and creeping grapevine (*V. flexuosa*, ---) in response to *Colletotrichum acutatum* (*Ca*) and *C. gloeosporioides* (*Cg*). Vertical bars represent standard error of means ($n = 3$)

CAT and HSP is elevated even in APX1-deficient Arabidopsis during light stress (Pnueli et al. 2003).

In this study, the expression of SOD showed different patterns in the leaves and immature and mature fruits of grapevines inoculated with the two pathogens. The SOD expression pattern varied and was dependent on the type of plant or external stresses (Zhou et al. 2017). It was previously reported that 10 genes of the SOD family show differential expression patterns in the roots, leaves, fruits, and seeds of grapevines against abiotic stresses, such as low temperature, high temperature, and salt stress (Hu et al. 2019). In our study, the expression level of CAT was higher than that of APX in 'Campbell Early' and creeping grapevines inoculated with pathogens. CAT and APX have been reported to be expressed differentially in response to biotic stress and abiotic stress in plants (Guan et al. 2018; Mano et al. 2001). By contrast, Haider et al. (2019) reported that the expression level of APX is higher than that of CAT in response to salt stress in grapevines.

3.4 Accumulation of hydrogen peroxide and HSF gene expression

To elucidate the relation between the expression of HSF genes and the generation of hydrogen peroxide in grapevines, HSF expression and the amount of ROS generation were investigated in the leaves and fruits of creeping grapevine inoculated with pathogens.

Among the selected HSFs, the expression of HSF11, HSF12, and HSF16 genes in the leaves and HSF14 and HSF16 genes in immature and mature fruits directly increased with increasing accumulation of hydrogen peroxide in creeping grapevine inoculated with *C. acutatum*. Moreover, a proportional correlation was observed between the amount of hydrogen peroxide and the expression of

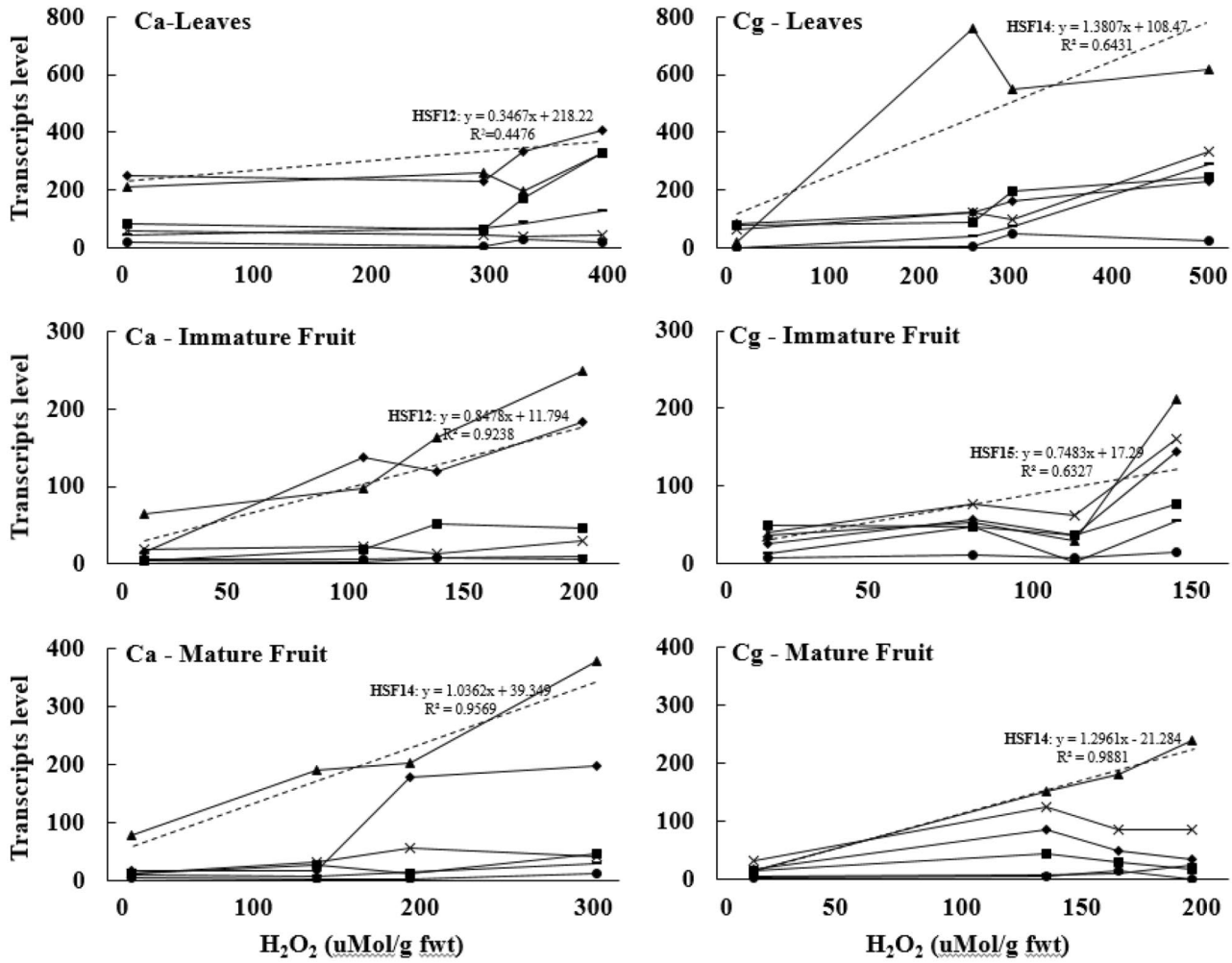

Fig. 7 Correlation between hydrogen peroxide content and expression levels of HSF11(■), HSF12 (⬥), HSF1 4(▲), HSF15 (x), HSF16 (-), and HSF17 (●) in leaves and immature and mature fruits of creeping grapevine (*V. flexuosa*) in response to *Colletotrichum acutatum* (*Ca*) and *C. gloeosporioides* (*Cg*)

HSF12 and HSF16 genes in leaves, the HSF14 gene in immature fruit, and the HSF14 and HSF16 genes in mature fruits in creeping grapevine inoculated with *C. gloeosporioides* (Fig. 7).

Previous studies reported that ROS accumulation induces the expression of several genes involved in the expression of HSF genes in plants (Miller and Mittler 2006; Volkov et al. 2006). Among the HSFs, it was reported that expression of HSF4 is associated with the generation of ROS in various plants, such as Arabidopsis (Gechev et al. 2005). In addition to external abiotic stresses (including high temperature), it is known that ROS accumulation also induces the expressions of HSF genes in plants (Davletova et al. 2005). Previous reports indicated that HSP is induced by the exogenous treatment of H_2O_2 in tomatoes and rice (Banzet et al. 1998; Lee et al. 2000). In Arabidopsis, HSFA2 was commonly expressed subsequent to H_2O_2 treatment and pathogen treatment, but HSF4 showed high expression levels only by pathogen infection and HSF6a was expressed by salt treatment (Miller and Mittler 2006).

4 Conclusion

In this study, variations of HSF gene expressions were observed in the leaves and immature and mature fruits of the two tested grapevines responding to foreign stresses. The expression of the selected HSFs (HSF11, HSF12, HSF14, HSF15, HSF16, and HSF17) was observed to be dependent on the increase of ROS in grapevines. Although the gene expression level of some HSFs was dependent on ROS accumulation, expression of other HSFs (HSF11, HSF13, and HSF17) increased without increasing hydrogen peroxide concentration in creeping grapevine responding to biotic and

abiotic stresses. However, the HSF gene expression through ROS accumulation was observed to be differentially regulated by the type of stress and variety in species of plants. In the pathway of HSF expression in response to pathogen infections, it was confirmed that regulation is directly associated and occurs via the activation of the hydrogen peroxide accumulation pathway. However, because there would be complicated pathways in HSF gene expression even in ROS accumulation, and in direct induction of their expression without ROS, further research is required on the various pathways for HSF gene expression in grapevines.

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Authorship contribution statement Young Jun You performed the overall experiment and data analysis. Soon Young Ahn performed experiment to confirm the results and wrote manuscript together. Hae Keun Yun designed and managed whole experiments and finalized the manuscript.

Declarations

Conflict of interest The authors declare that they have no conflict of interests.

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